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Relative Roles of Metabolism and Renal Excretory Mechanisms in Xenobiotic Elimination by Fish

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Renal clearance techniques were used to examine the relative contributions of metabolism and renal tubular transport in determining the rates of excretion of benzo(*a*)pyrene (BaP) and several of its phase I metabolites by southern flounder, *Paralichthys lethostigma*. Each compound (^{3}H-labeled) was injected at a dose of 2.5 μmole/kg, producing plasma concentrations of 1 to 5 μM. Despite extensive plasma binding (>95%), the uncorrected renal clearance of BaP-7,8-dihydrodiol exceeded the glomerular filtration rate (GFR) by more than 20-fold. Phenolic BaP metabolites also showed net secretion (1.5- to 3-fold). At times prior to 3 hr, BaP itself showed an average clearance of only 0.2 times the GFR. After 3 hr, BaP clearance increased to three times the GFR. Decreasing the dose of BaP injected also dramatically increased its clearance. Clearances of all four compounds studied were reduced by probenecid and other organic anion, including the herbicide 2,4-dichlorophenoxyacetic acid. HPLC analysis demonstrated that the bulk of the material excreted in the urine was not the parent compound, but sulfate or glucuronide conjugates of its phenolic or dihydrodiol metabolites. Excretion of sulfate conjugates predominated over the first 24 hr, whereas the glucuronide conjugates were the primary excretory products in succeeding days. *In vitro*, isolated renal tubules transported both glucuronide and sulfate conjugates, but sulfates were the preferred substrates. Isolated tubules were shown to be capable of catalyzing conjugation reactions, producing predominantly glucuronide conjugates. Liver slices produced both types of conjugates. Thus, the rapid excretion of BaP-7,8-dihydrodiol reflected a combination of two processes. First, this metabolite was rapidly converted to its sulfate conjugate, primarily via extrarenal tissues. Second, the sulfate conjugate was preferentially transported to the urine via secretion on the organic anion transport system.

Introduction

Aquatic organisms are exposed to a wide variety of foreign chemicals. Many of these chemicals are potentially toxic to the organisms themselves and to other animals, including man, which may feed on them. Furthermore, once within the aquatic organism, a number of these chemicals, e.g., the polycyclic aromatic hydrocarbons (PAHs), may be converted to still more toxic forms through metabolic oxidation, usually via the cytochrome P-450 monooxygenase system (*1*). Therefore, the primary focus of much of the work in this area has been on mechanisms of metabolic activation and on the enzyme systems, e.g., epoxide hydrolase and the glutathione transferases, which mediate detoxication of the activated, chemically reactive metabolites (*2,3*).

Much less attention has been given to the second means of reducing tissue concentrations of potentially toxic compounds, elimination from the body via urinary and biliary excretion. Certainly, metabolic studies in fish, as in mammals, amply demonstrate conversion of lipophilic xenobiotics to more polar forms. Conversion to lipophilic xenobiotics alone should accelerate elimination of toxic compounds (*4,5*). Indeed, the appearance of numerous polar xenobiotic metabolites in urine and bile has been widely documented (*6–8*). However, these studies have not addressed the possibility that different phase I metabolites might be excreted differently. For example, although the model PAH, benzo(*a*)pyrene (BaP), is known to be metabolized through a number of increasingly oxidized intermediates to its putative carcinogenic metabolite, BaP 7,8-*t*-dihydrodiol-9,10-epoxide, neither the extent of excretion nor the factors that regulate excretory rate are known for BaP or any of its metabolites. Clearly, such factors may play an important role in determining the retention and toxicity of PAHs to the marine organism itself and to those who consume such contaminated seafood.

In the studies reported in this paper, we have examined the urinary excretion of BaP by the southern flounder (*Paralichthys lethostigma*) and compared its excretion to that of several of its more polar metabolites, with particular attention to BaP-7,8-*t*-dihydrodiol (BaP-7,8-diol), an immediate precursor to the putative ultimate carcinogenic BaP metabolite, the 7-8-diol-9,10--epoxide. We also examined several of the processes that contribute to the pattern and rate of BaP-7,8-diol excretion. These studies were conducted using a marine teleost for two reasons.

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Table 1. Summary of typical clearance experiment showing the effect of probenecid on the clearance of BaP-7, 8-diol.[a]

Clearance period	Time, min	Urine flow, mL/hr	PEG			BaP-7,8-diol			Clearance ratio[b]
			Plasma, mg/mL[c]	Urine, mg/mL	Clearance, mL/hr	Plasma, nmole/mL	Urine, nmole/mL	Clearance, mL/hr	$C_{7/8}/C_{PEG}$
0	0–74	0.50[d]	0.05	0.12	1.15	0.91	10.42	5.73	4.97
2	75–88	0.50[d]	0.27	0.38	0.71	0.71	23.98	8.27	11.73
4	93–100	0.58	0.33	0.75	1.32	1.32	28.11	10.32	7.77
6	106–116	0.54	0.41	1.16	1.54	1.54	32.08	9.71	6.29
8	128–136	0.84	0.50	2.16	3.67	3.67	54.84	23.06	6.28
$X \pm$ SEM ($n = 9$; periods 1-9)	—	0.60[d] ± 0.14	0.38 ± 0.03	1.06 ± 0.25	1.98 ± 0.61	1.98 ± 0.61	34.28 ± 4.88	14.24 ± 3.90	9.08 ± 1.18
Probenecid									
10	143–147	0.87	0.55	1.77	2.80	2.08	33.02	13.87	4.96
12	151–156	0.69	0.57	1.40	1.69	2.10	32.74	10.71	6.35
14	171–180	0.52	0.63	2.25	1.89	2.12	25.32	6.25	3.36
16	188–194	1.02	0.72	3.05	4.33	2.13	21.81	10.47	2.42
18	204–228	0.50	0.78	3.77	2.18	2.18	17.06	3.91	1.62
$X \pm$ SEM ($n = 6$: periods 13–18)	—	0.64 ± 0.13	0.69 ± 0.03	2.76 ± 0.52	2.52 ± 0.52	2.14 ± 0.01	23.06 ± 1.79	6.84 ± 1.21	2.92 ± 0.05

[a]Fish weight, 616 g; doses: 250 mg/kg ^{14}C-PEG; 2.5 mole/kg ^{3}H-BaP-7,8-diol injected 50% IM, 50% IV at time 0; probenecid, 25 mole/kg IV at 136 min.
[c]Clearance ratio is independent of urine flow rate.
[b]Plasma values are those determined for the midpoint of the collection period.
[d]Urine flow was erratic during periods 0–2, estimated values based on average over first 90 min were used to calculated data for periods 0, 1, and 2. $n = 7$ for this mean (1 and 2 excluded).

First, results from such studies should shed some light on the dynamics of BaP and metabolites in an important class of aquatic food organisms. Second, the marine teleost presents several distinct experimental advantages for examining the role of renal excretory processes in xenobiotic elimination (*9*). For example, because the marine teleost has an extensive renal portal system, the tubules are bathed in a blood flow that approaches the entire cardiac output. This anatomical arrangement allows maximal impact of the kidney on the excretion of all solutes present in the circulation. It also permits direct experimental access to the blood perfusing the kidney, since the readily accessible caudal vein feeds directly into the renal portal system. Thus, application of *in vivo* renal clearance techniques to marine teleosts can provide a great deal of information about the renal handling of a given agent, particularly the contribution of secretory tubular transport toward its net elimination (*9*). Furthermore, the tubules themselves may be readily isolated for *in vitro* examination of their metabolic capacity and transport properties (*9,10*). As shown here, these studies demonstrate that BaP and its phase I metabolites are excreted into the urine at different rates. These differences may be ascribed in large part to the combined action of phase II metabolic events and the renal organic anion secretory transport system mediates their excretion.

Materials and Methods

Animals

Adult southern flounder (0.5 to 3.0 kg) of both sexes were netted in the wild in the vicinity of Matanzas Inlet, Florida. These fish were maintained in the laboratory in flowing, sand-filtered sea water for not less than 1 week following capture. Live shrimp or juvenile fish were continuously available as food, approximating the diet of these fish in the wild.

Renal Clearance Determination

Clearance measurements were carried out as previously described (*11*). The glomerular filtration rate (GFR) marker, ^{14}C-polyethyleneglycol (PEG) (molecular weight 4000; dose 250 mg/kg) and the ^{3}H-labeled BaP or BaP metabolite (2.5 μmole/kg) were injected together 1 hr prior to the first collection period. One-half of the dose was administered IV via the caudal vein as a priming dose and the remainder was given IM in the earlier work (*11*). Resulting plasma concentrations were 0.5 to 1.0 mg/mL for PEG and 1 to 5 μM for BaP and metabolites and were stable over the period from 1 to 6 hr after injection. A typical experiment with BaP-7,8-diol is shown in Table 1.

In each experiment, clearances were calculated based on radioactivity in plasma and urine in the usual manner, i.e., the quantity of label appearing in the urine per unit time was divided by the plasma concentration of label. Calculated clearance values were not corrected for plasma binding (> 95% for BaP and each of its metabolites, as determined by ultrafiltration; data not shown). Thus, the values presented represent minimal estimates of renal clearance. This choice was based on several important considerations. First, because the concentrations of free compounds were so low, small errors in binding measurements would be grossly magnified in the final clearance calculations. Furthermore, it is not yet established how effectively tubular secretory transport may compete with plasma binding sites for access to these compounds. Thus, we have chosen the conservative course of estimating tubular transport based on the assumption that all labeled BaP or metabolite was available for transport. Our second assumption was to use the clearance of label to estimate the clearance of each parent compound. We know that each compound was subject to extensive metabolism

during the course of these *in vivo* studies. However, the important initial point was to focus on the relative rates of excretion for all compounds derived from a given parent molecule and to determine whether evidence could be obtained for the participation of tubular transport in the excretion process. As the data presented below clearly attest, differences in clearance rate and net tubular transport were readily apparent between BaP and its metabolites.

HPLC Analysis

Urine samples were prepared for analysis using a Sep-Pak (C-18 reverse phase). Urine (1.5 mL) was passed through the Sep-Pak, which was then washed with 5 mL of water, followed by 5.0 mL of 25% methanol. The sample was eluted with 5 mL of 100% methanol, evaporated to dryness, and dissolved in 200 μL of 100% methanol; 50 μL of this material was injected into the HPLC system (Altex Model 420). Initial analysis was performed using a Waters radial pack C-18 column eluted at 1 mL/min with 6 mL of 60% methanol:water, followed by 9 mL of 90% methanol:water. Standards eluted from this column in the following order: BaP-3-SO_4 (4 min), BaP-3-glucuronide (5 min), BaP-7,8-diol (11 min), and BaP-3-OH (13.5 min).

All later work was performed using an Altex ultrasphere ODS 5 μm C-18 reverse phase column. Conditions used were a flow rate of 0.75 mL/min with 45% methanol:water (9 mL), 95% methanol:water (8.25 mL) and return to 45% methanol (27.75 mL). Standards eluted in the following order: BaP-3-glucuronide (7.5 min), BaP-3-SO_4 (11 min), BaP-7,8-diol (26 min), BaP-3-OH (29.4 min). Although the order of elution was altered, the relative abundance of conjugates was unchanged. Identities of the two conjugated metabolites were confirmed by enzymatic hydrolysis with β-glucuronidase and sulfatase according to the methods of Varanasi and Gmur (*5*).

Isolated Tubule and Slice Studies

Tubules were prepared from flounder kidney as originally described by Forster (*10*) with minor modifications (*12*). Approximately 10 mg of tissue was incubated in 2 mL of oxygenated Tris Forster's saline (in mM; Na 140, K 2.5, Ca 1.5, Mg 1, Cl 147.5, and Tris 20 at pH 8.25) containing labeled BaP or BaP metabolite. After the desired incubation time, the tubules were blotted on filter paper, weighed, and homogenized in 0.5 mL of distilled water. Acetone (0.5 mL) was added, and the mixture was extracted three times with 4 mL of ethyl acetate. The ethyl acetate extract was evaporated to dryness under a stream of dry nitrogen, and the residue was taken up in methanol for HPLC analysis as above. Liver slices (0.5 mm thick) were prepared using a Stadie-Riggs microtome. Incubation and processing procedures for slices were identical to those described above for renal tubules.

In experiments to compare the tubular transport of glucuronide and sulfate conjugates, 4-methylumbelliferone (4-MU) and its conjugates were used as substrates. The isolated tubules were incubated as before with 10 μM 4-MU or 4-MU conjugate. After incubation, the tissue was weighed and rapidly denatured by heating with 1 mL of 0.2 M acetate buffer (pH 5.0) in a boiling water bath for 10 min. (4-MU and its conjugates were stable for at least 60 min at 100°C; data not shown.) The tubules were then homogenized. To determine 4-MU itself, another 1 mL of acetate buffer was added, and this mixture was extracted with 2.0 mL of ethyl acetate. Samples were dried and taken up in methanol for analysis by HPLC with fluorescence detection. 4-MU sulfate and glucuronide were enzymatically hydrolyzed prior to extraction with ethyl acetate and analysis as for 4-MU itself. Conditions used for hydrolysis were as described above for BaP conjugates. Correction for recovery of 4-MU and its conjugates was determined by analysis of known quantities of each compound performed in parallel with each group of unknowns. Recoveries ranged from 65 to 80%.

Chemicals

Both labeled and unlabeled BaP metabolites were obtained from the Cancer Research Program of the National Cancer Institute. 4-MU and its glucuronide and sulfate conjugates, β-glucuronidase and sulfatase, were purchased from Sigma Chemical Company (St. Louis, MO). All other chemicals were of the highest purity available from commercial sources.

Results

Renal Clearances

In preliminary experiments, ^{3}H-BaP and ^{14}C-PEG (as GFR marker) were injected IM. As in previous studies (*12*), the more water soluble PEG was effectively absorbed into plasma from muscle and reached a stable plasma concentration within 1 hr. However, the more lipophilic BaP was not effectively absorbed. Therefore, in the experiments reported here, BaP was presented by IV injection into the caudal vein. The more water soluble BaP metabolites were presented in two doses, a priming dose of 1.25 μmole/kg injected IV and a maintenance dose of 1.25. μmole/kg injected IM. As shown in Tables 1 and 2, these protocols yielded relatively stable plasma concentrations of BaP and its metabolites. Note, however, that plasma concentrations following IV injection of ^{3}H-BaP were somewhat higher than those obtained following IV plus IM injection of its phenolic and dihydrodiol metabolites, reflecting both the slower excretion rate for BaP and its route of administration.

Two major points should be noted when comparing the renal handling of BaP with that of its phenolic and dihydrodiol derivatives (Table 2). First, average clearance values over the first 3 hr after injection of labeled BaP were substantially lower than the GFR, demonstrating net reabsorption. However, upon examination of the final urine-to-plasma ratios for BaP and PEG, it was clear that by the end of the 4 to 6 hr experiment, label injected as BaP had begun to appear in the urine at a rate more rapid than the GFR marker, i.e., net secretion was taking place. In fact, BaP clearances measured at times exceeding 3 hr after injection averaged more than 10 times the earlier clearance values. Furthermore, as shown in Table 3, it was possible to dramatically increase the renal clearance of ^{3}H-label injected as BaP by reducing the quantity of BaP injected. Such changes in renal handling would be most readily explicable by metabolic conversion of BaP to a form, or forms, subject to active tubular transport. In the first instance, increased metabolism followed increased contact time. In the second case, lowering the total amount of BaP injected could have increased the relative contribution of metabolites in the total mix of BaP-derived compounds. Indeed, as discussed below, this appears to be the case.

Table 2. Renal handling of BaP and potential metabolites by the southern flounder.[a]

Measurement	BaP, <3 hr	BaP, >3 hr	1-OH-BaP	7-OH-BaP	7,8-Diol
Plasma concentration, nmole/mL	7.1 ± 1.1	6.4 ± 1.4	1.1 ± 0.3	2.3 ± 0.45	0.7 ± 0.2
Urine concentration, nmole/mL	2.4 ± 1.0	70.4 ± 4.5	4.9 ± 2.2	6.4 ± 1.6	17.1 ± 5.1
Clearance[b] mL/hr	0.2 ± 1.0	3.8 ± 0.5	0.9 ± 0.3	0.7 ± 0.2	15.2 ± 5.1
Glomerular filtration rate, mL/hr	0.8 ± 0.1	1.2 ± 0.3	0.9 ± 0.3	0.8 ± 0.3	0.8 ± 0.2
Ratio C_x/GFR[c]	0.2 ± 0.1	3.4 ± 0.4	1.8 ± 0.6	1.8 ± 0.9	22.7 ± 3.9
n[d]	3	2	6	4	9

[a]Dose was 2.5 μmole/kg for all compounds. The glomerular filtration rate was measured simultaneously using PEG (molecular weight 4000).

[b]The clearance (C_x) is calculated as $(U_x * V)/P_x$ and is the volume of plasma cleared of compound x per unit time. U_x, concentration of x in urine; P_x, concentration of x in plasma; and V, rate of urine production.

[c]The ratio of C_x/GRF will be greater than 1 only for compounds added to the urine by the renal tubules (i.e., secreted). A ratio less than 1 means the compound was removed by the tubules (i.e., reabsorbed). GFR, glomerular filtration rate.

[d]Multiple clearance measurements were made in each animal. Thus, total clearance periods for each group were 15 (BaP < 3 hr); 6 (BaP) > 3 hr); 45 (1-OH-BaP); 30 (7-OH-BaP); and 63 (7,8-diol).

Despite evidence for tubular secretion of BaP late (> 3 hr) in the experiments (Table 2) or after a low dose of BaP (Table 3), it was nevertheless clear that BaP phenols and dihydrodiols were cleared by the kidney far more rapidly than BaP itself immediately after injection (Table 2). The phenolic products, 1-OH-BaP and 7-OH-BaP, were intermediate in their clearance rates. In most fish, the mean clearance ratio was greater than unity, demonstrating net tubular secretion. By far, the most effectively eliminated derivative examined was BP-7,8-diol, which was cleared on the average 10 to 30 times the GFR. For this reason, subsequent experiments focused largely on BaP-7,8-diol, an important metabolite of BaP.

The influence of altered temperature and of enzyme induction, both of which should alter metabolic rate, were assessed in fish given either BaP or BaP-7,8-diol. To examine the effects of temperature, fish were injected with ^{3}H-BaP-7,8-diol in winter when the water temperature was 12 to 14 °C, about −10 °C lower than the water temperatures present during the initial clearance studies (in summer). Clearance of the diol fell to only one-tenth of the summer values. On the other hand, injection with 3-methylcholanthrene (10 mg/kg) 1 week prior to clearance determination, previously shown to increase metabolism via induction of cytochrome P-450 in this species (*13*), enhanced the renal excretion of BaP but was without significant effect on BaP-7,8-diol clearance (Table 4). These results suggest that the excretion of BaP requires oxidative metabolism but that excretion of the dihydrodiol does not. However, dihydrodiol excretion was temperature dependent, indicating that a subsequent metabolic step, e.g., conjugation, and/or the excretory process itself, was temperature dependent.

Table 3. Influence of dose on clearance of BaP by southern flounder.

Measurement	BaP 2.5 μmole/kg	BaP 0.25 μmole/kg
Plasma concentration, μM	7.5 ± 1.2	0.5 ± 0.2
Urine concentration, μM	10.8 ± 5.6	11.1 ± 3.8
Clearance, mL/hr	1.1 ± 0.4	10.4 ± 2.0
Clearance BaP/GFR[a]	0.8 ± 0.4	17.2 ± 9.1
n, fish	5	4
n, clearance periods	58	39

[a]GFR, glomerular filtration rate.

Therefore, we examined the secretory mechanism and the sensitivity of excretion rate to manipulation of the secretory process. Table 1 and Figure 1 demonstrate the sensitivity of renal excretion to inhibition of BaP-7,8-diol excretion to inhibition of the organic anion transport system by probenecid, the classical inhibitor of this system. Probenecid also reduced the clearances of both BaP 1- and 7-phenols (not shown). In addition, both the renal (Table 5) and hepatic (not shown) accumulation of these phenols was markedly reduced by probenecid, as would be predicted if their accumulation and subsequent excretion were mediated by the organic anion transport system known to be present in both organs.

Other organic anion, notably the anionic herbicide, 2,4-dichlorophenoxyacetic acid (2,4-D), were also effective inhibitors of the renal excretion of BaP-7,8-diol. As shown in Figure 2*A*, administration of a small dose (2.5 μmole/kg) of 2,4-D led to a prompt, but transient inhibition of BaP-7,8-diol clearance. As shown previously (*6*), 2,4-D is an excellent substrate for the organic anion system and is excreted very rapidly. Thus, the transient nature of the inhibition seen here apparently reflects elimination of the inhibitor. When a higher dose of 2,4-D (μmole/kg) was given prior to the administration of BaP-7,8-diol (Fig. 2*B*), inhibition was much more extensive

Table 4. Effect of environmental temperature and enzyme induction on the renal clearance of BaP or BaP-7,8-dihydrodiol.

Measurement	BaP Control	BaP Induced	BaP-7,8-diol Control	BaP-7,8-diol Induced	BaP-7,8-diol Low temperature
Plasma concentration, μM	7.1 ± 1.1	4.8 ± 2.6	0.7 ± 0.2	1.6 ± 0.3	1.6 ± 0.6
Urine concentration, μM	2.4 ± 1.0	7.0 ± 3.6	17.1 ± 5.1	38.7 ± 14.4	4.7 ± 0.1
Clearance, mL/hr	0.2 ± 0.1	4.6 ± 2.4	15.2 ± 4.2	13.6 ± 3.0	1.5 ± 1.0
n	3	4	9	4	2
Total periods	15	60	63	45	8

Table 5. Effect of probenecid on the renal accumulation of BaP metabolites by the southern flounder.[a]

BaP metabolite	Plasma concentration, μM	Renal concentration, μM	Tissue/plasma
Control			
7,8-Diol	0.7 ± 0.2	6.0 ± 0.8	8.6
1-OH	1.1 ± 0.3	15.1 ± 7.0	13.7
7-OH	2.3 ± 0.4	33.0 ± 0.9	14.3
Probenecid			
7,8-Diol	1.1 ± 1.0	1.9 ± 0.5*	1.7
1-OH	0.5 ± 0.1	0.7 ± 0.1	1.4
7-OH	4.8 ± 2.2	10.8 ± 3.3*	2.2

[a]Plasma and renal concentration are means ± SEM for four to nine control animals and two probenecid-treated animals. Mean values were used to calculate tissue accumulation ratios, i.e., tissue concentration/plasma concentration.

*$p < 0.05$ versus control.

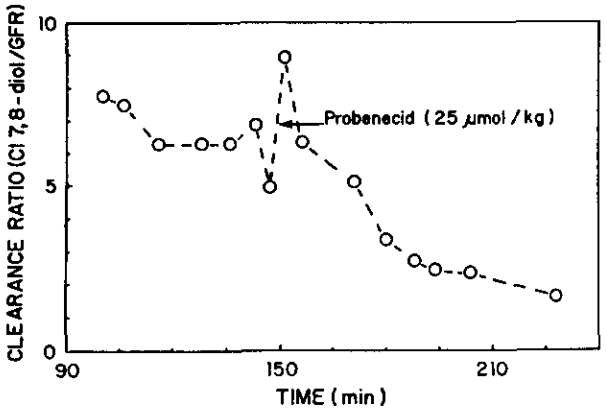

FIGURE 1. Inhibition of the renal clearance of BaP-7,8-diol by probenecid. Net secretion of BaP-7,8-diol, expressed as the ratio of BaP-7,8-diol clearance over the glomerular filtration rate (GFR) was followed for 2.5 hr. Probenecid was administered IV at 155 min, and the clearance ratio was followed for 90 additional min. Over this interval, the clearance ratio was reduced dramatically, and net renal secretory transport of BP-7,8-diol was nearly abolished.

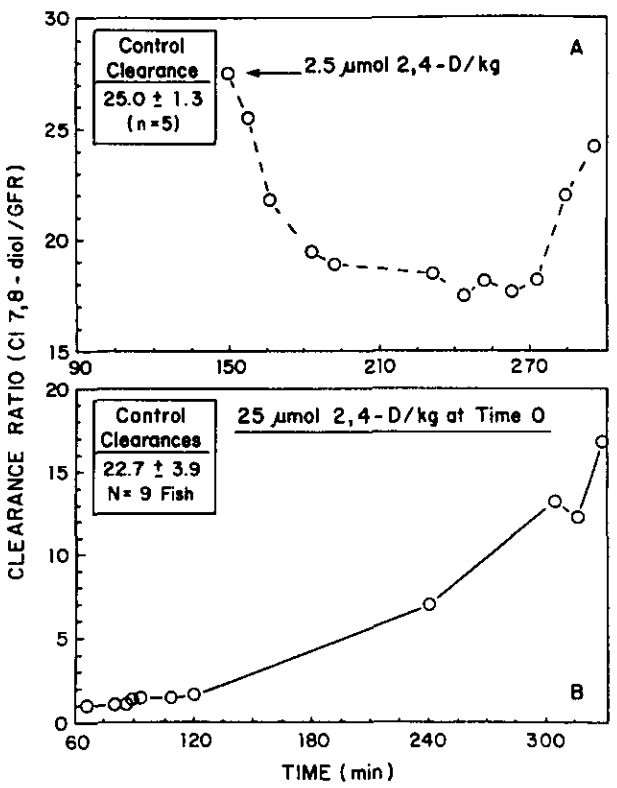

FIGURE 2. Inhibition of renal secretory transport of BaP-7,8-diol by the herbicide 2,4-dichlorophenoxyacetic acid (2,4-D). (*A*) A single dose of 2,4-D (2.5 μmole/kg) was administered 150 min after an identical dose of BaP-7,8-diol. Clearance fell abruptly from control values in that fish of 25 times the GFR to approximately 18 times the GFR and largely recovered in 2.5 hr. (*B*) 2,4-D (25 μmole/kg) was given simultaneously with ^{3}H-BaP-7,8-diol (2.5 μmole/kg). Inhibition was profound, i.e., secretion was almost totally blocked for the first 2 hr and had not reached control values after more than 5 hr.

(> 95% over the first 2 hr), and clearance values were still substantially below control after more than 6 hr.

Given the importance of metabolism in determining the efficacy of elimination and transport, we examined the chemical nature of the labeled material appearing in the urine using HPLC techniques. As shown in Figure 3, the bulk of the radioactivity administered as BaP-7,8-diol appeared in the urine as a mixture of conjugated metabolites. On the first day after injection of ^{3}H-7,8-diol, the largest fraction of the excreted label co-chromatographed with the sulfate conjugate standard. The glucuronide conjugate was also prominent; smaller portions were excreted as unchanged BaP-7,8-diol. In this flounder (Fig. 3), some label also chromatographed with the phenolic standard, but this peak was missing in the urine of other fish. After the first day, the portion of the label excreted as the sulfate conjugate decreased markedly and was nearly undetectable on day 3. The identities of the several radioactive peaks were confirmed by enzymatic hydrolysis (Fig. 4). Treatment of the urine collected within the first day after 7,8-diol injection (high in putative sulfate conjugate) with sulfatase essentially eliminated the sulfate peak (Fig. 4). Similarly, treatment of the urine collected on days 2 and 3 with glucuronidase abolished the putative glucuronide peak.

The rapid appearance and elimination of the sulfate conjugate of BaP-7,8-diol might reflect its preferential transport on the organic anion system. This possibility could not be assessed directly due to the unavailability of sufficient quantities of

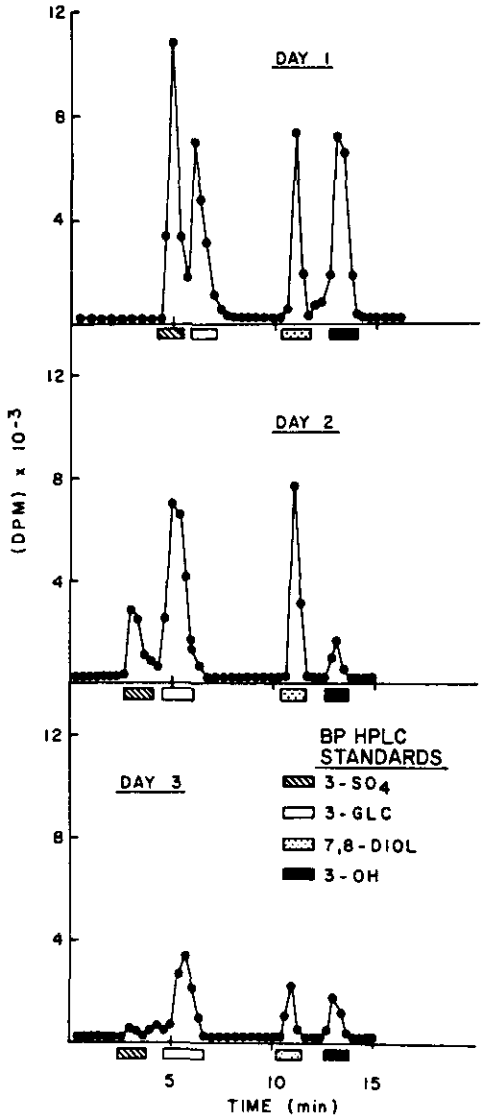

FIGURE 3. HPLC analysis of flounder urine on days 1 through 3 after injection of ^{3}H-BP-7,8-diol (2.5 μmole/kg) to intact fish. The elution position of known standard BaP metabolites run concurrently with the urine samples is shown below the x-axis of each trace.

labeled 7,8-diol conjugates. However, 4-MU and its glucuronide and sulfate conjugates were commercially available and could be readily assayed fluorimetrically. Thus, we were able to expose isolated flounder tubules to 4-MU or its conjugates *in vitro* and determine the relative effectiveness of tubular transport for the three classes of substrate. As shown in Table 6, each of these xenobiotics was accumulated by the flounder renal tubules. The accumulation of 4-MU itself was the most modest and reflected both entry of the parent molecule and subsequent conversion to conjugates. Therefore, accumulation of 4-MU is a maximal estimate of transport activity. Both 4-MU-glucuronide and 4-MU-sulfate were accumulated to a much greater extent than 4-MU itself. Furthermore, the accumulation of conjugates was largely blocked by probenecid, which inhibits the organic anion transport system, and by cyanide, which should inhibit active transport through blocking cellular respiration and ATP production. As suggested by the *in vivo* results with BaP-7,8-diol, the sulfate conjugate yielded cellular accumulation of approximately twice that of the glucuronide conjugate. Thus, it would appear that the pattern of excretion documented above does indeed reflect the secretory transport of both conjugates. The sulfate conjugate appeared in the greatest quantities initially and was eliminated first. The glucuronide continued to be transported and eliminated over subsequent days.

Finally, we examined the ability of kidney and liver to produce these conjugated metabolites from BaP-7,8-diol. Isolated renal tubules or liver slices were incubated in Forster's saline with 10 μM ^{3}H-BaP-7 8-diol for 4 (kidney) to 6 (liver) hr *in vitro*. As shown in Table 7, both tissues produced sulfate and glucuronide conjugates. However, there were several differences between the two tissues. Most notably, the kidney tubules were far more active *in vitro*, converting nearly 50% of the substrate to conjugates within 4 hr. In contrast, liver slices only produced about 20% conjugates after 6 hr of incubation. The pattern of metabolism was also quite different, with the kidney tubules producing more than 10 times as much glucuronide as sulfate conjugates. The liver also produced more glucuronide conjugate, but the ratio was less than 3 to 1. If the *in vitro* experiments reflect *in vivo* metabolism of BaP-7,8-diol, it would appear that the bulk of the sulfate conjugate appearing in the urine during the first 24 hr after

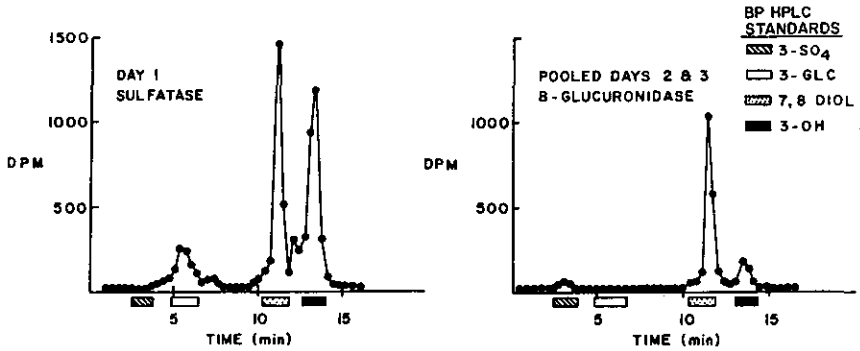

FIGURE 4. Enzymatic hydrolysis of BaP-7,8-diol metabolites present in flounder urine after injection of 2.5 μmole/kg ^{3}H-BP-7,8-diol. Samples selected for analysis were day 1 urine (high in putative sulfate conjugate, Fig. 3) and pooled urine from days 2 and 3 (high in putative glucuronide conjugate, Fig. 3). Other details were as described in the legend of Figure 3.

Table 6. *In vitro* transport of 4-MU and its conjugates by isolated tubules from flounder kidney.[a]

Compound	Tissue concentration, nmole/g	Tissue concentration/medium concentration		
		Control	+Probenecid, 1 mM	+Cyanide, 1 mM
4-MU	22.4	2.2	—	—
4-MU-glucuronide	47.7	4.8	0.5	0.6
4-MU-sulfate	72.6	7.3	1.9	1.6

[a]Tubules were incubated with 10 μM 4-MU or its sulfate or glucuronide conjugate for 60 min *in vitro*. Tissue content was assayed fluorometrically as 4-MU by HPLC after enzymatic hydrolysis.

Table 7. Metabolism of BaP-7,8-dihydrodiol by southern flounder kidney tubules and livers slices *in vitro*.[a]

Organ	Sulfate	Glucuronide	7,8-Diol	Unknown
Kidney	4.0 ± 2.6	43.8 ± 2.9	47.8 ± 3.0	4.4 ± 3.4
Liver	6.6 ± 1.0	14.1 ± 2.2	71.1 ± 2.2	8.2 ± 1.1

[a]Results are presented as percent label in each form. Values are given as means ± SEM for four fish. Incubation times were 4 hr for kidney and 6 hr for liver.

injection must have been produced elsewhere in the body and been transferred to the kidney via the circulation. This conclusion is also supported by the ability of other organic anions (probenecid and 2,4-D) to inhibit both renal secretion of BaP-7,8-diol conjugates (Table 1, Figs. 1 and 2) and to reduce the accumulation of conjugates both *in vivo* (Table 5) and *in vitro* (Table 6).

Discussion

In any attempt to assess the potential impact of chemically contaminated seafood on human consumers, one must be able to assess not only the degree of contamination, but also the retention of that contamination within the food organism for subsequent transfer up the food chain. The results reported above demonstrate that several factors including oxidative metabolism, conjugation reactions, and excretory mechanisms play important roles in determining both the retention and the potential toxicity of foreign chemicals.

For the many lipophilic xenobiotics such as BaP, it has been recognized for years that oxidative metabolism via cytochrome P-450 plays an important dual role (*1*). On one hand, oxidative metabolism increases water solubility of these xenobiotics, thus increasing their plasma concentrations and making them more readily available for urinary and/or biliary excretion. On the other hand, a significant number of these oxidative metabolites are biologically active in their own right, often exceeding their parent in reactivity and toxicity. Therefore, phase II reactions, which include epoxide hydrolase, glutathione transferase, and conjugation reactions with glucuronic acid, sulfate, or amino acids, normally assume critical roles in terminating the toxicity of these xenobiotics and their phase I metabolites by converting them to nontoxic products that are readily excreted. What has been demonstrated in these studies, as emphasized in Table 2, is that the different products of oxidative metabolism are also handled in very specific and different ways by the available renal excretory mechanisms. Furthermore, as shown in Table 6, different conjugated metabolites (e.g., glucuronides versus sulfates) are also subject to differential handling by the renal transport system that mediates their elimination. Thus, the overall retention and the potential toxicity of a given xenobiotic are determined by the specific combination of the actions of phase I/phase II metabolic pathways and excretory mechanisms on that compound.

For BaP and the specific metabolites studied here, the factors discussed here sort out in the following manner. *a*) Increased oxidative metabolism accelerates the elimination of BaP itself, as evidenced by the increased rate of excretion of BaP at later times after injection into the fish (Table 2), the accelerated loss of BaP-derived radioactivity when the injected dose was reduced (Table 3), and the increased excretion at higher environmental temperatures or after induction of the cytochrome P-450 monooxygenase system by 3-methylcholanthrene (Table 4). *b*) In contrast to the result for BaP itself, BaP-7,8-diol excretion was not significantly changed by P-450 induction (Table 4), implying that subsequent steps, e. g., conjugation and renal secretory transport, were more critical for dihydrodiol excretion. *c*) The accelerated excretion of all four labeled BaP compounds depended upon their secretory transport on the organic anion system, as evidenced by the inhibition of transport *in vivo* (Table 1, Fig. 1) and *in vitro* (Table 6) by probenecid and 2,4-D (Fig. 2). *d*) Sulfate-conjugated BaP metabolites were better substrates for renal organic anion transport than comparable glucuronide conjugates *in vitro* (Table 6) and *in vivo* (Figs. 3 and 4). *e*) The ability of the herbicide 2,4-D to reduce the rate of elimination of BaP-7,8-diol (Fig. 2) demonstrates the possibility that the simultaneous presence of two or more substrates for the organic anion system may retard the elimination of toxic xenobiotics such as BaP-7,8-diol, increasing the likelihood of both systemic toxicity in the fish and enhanced transfer of xenobiotic to subsequent consumers, including man. *f*) Although both kidney and liver were shown to be capable of conjugating BaP metabolites (Table 7), the bulk of the excreted metabolites must have been produced outside the kidney, as evidenced by the reduced renal tissue levels and excretion following inhibition of the organic anion transport system by probenecid or 2,4-D (Tables 1 and 5; Figs. 2 and 3).

In conclusion, these data emphasize that important interactions occur between metabolism and excretory mechanisms that together determine the retention of potentially toxic chemicals by fish. Without an understanding of both processes, it is not possible to predict either the extent of accumulation or the toxicity of the retained products. Thus, any attempt to address the potential toxicity of such chemicals in the exposed organism or in subsequent consumers requires a thorough knowledge of both processes.

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